ELSEVIER

Aquatic Toxicology 82 (2007) 227–241

AQUATIC TOXICOLOGY

www.elsevier.com/locate/aquatox

Identification of gene expression indicators for thyroid axis disruption in a *Xenopus laevis* metamorphosis screening assay Part 1. Effects on the brain

Caren C. Helbing [a,*], Carmen M. Bailey [a], Lan Ji [a], Mark P. Gunderson [a], Fang Zhang [a], Nik Veldhoen [a], Rachel C. Skirrow [a], Ruixia Mu [b], Mary Lesperance [b], Gary W. Holcombe [c], Patricia A. Kosian [c], Joseph Tietge [c], Joseph J. Korte [c], Sigmund J. Degitz [c]

[a] *Department of Biochemistry and Microbiology, P.O. Box 3055, Stn. CSC, University of Victoria, Victoria, British Columbia, V8W 3P6, Canada*
[b] *Department of Mathematics and Statistics, University of Victoria, Victoria, British Columbia, V8W 3P4, Canada*
[c] *U.S. Environmental Protection Agency, Mid-Continent Ecology Division, 6201 Congdon Blvd., Duluth, MN 55804, USA*

Received 19 December 2006; received in revised form 1 February 2007; accepted 3 February 2007

Abstract

Thyroid hormones (TH), thyroxine (T_4) and 3,5,3′-triiodothyronine (T_3), play crucial roles in regulation of growth, development and metabolism in vertebrates and their actions are targets for endocrine disruptive agents. Perturbations in TH action can contribute to the development of disease states and the US Environmental Protection Agency is developing a high throughput screen using TH-dependent amphibian metamorphosis as an assay platform. Currently this methodology relies on external morphological endpoints and changes in central thyroid axis parameters. However, exposure-related changes in gene expression in TH-sensitive tissue types that occur over shorter time frames have the potential to augment this screen. This study aims to characterize and identify molecular markers in the tadpole brain. Using a combination of cDNA array analysis and real time quantitative polymerase chain reaction (QPCR), we examine the brain of tadpoles following 96 h of continuous exposure to T_3, T_4, methimazole, propylthiouracil, or perchlorate. This tissue was more sensitive to T_4 rather than T_3, even when differences in biological activity were taken into account. This implies that a simple conversion of T_4 to T_3 cannot fully account for T_4 effects on the brain and suggests distinctive mechanisms of action for the two THs. While the brain shows gene expression alterations for methimazole and propylthiouracil, the environmental contaminant, perchlorate, had the greatest effect on the levels of mRNAs encoding proteins important in neural development and function. Our data identify gene expression profiles that can serve as exposure indicators of these chemicals.

Keywords: cDNA array; Quantitative real time polymerase chain reaction; Amphibian; Frog; Metamorphosis

1. Introduction

As part of the mandate of the Food Quality Protection and Safe Drinking Water Acts established by the US Congress in 1996 and in response to the recommendations of the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), the US Environmental Protection Agency (US EPA) is developing and implementing a screening approach for thyroid axis disruption using metamorphosis in the *Xenopus laevis* frog tadpole (EPA, 1998). This assay takes advantage of the dependence of amphibian metamorphosis on normal thyroid function and the use of whole animals to allow consideration of physiology, chemical biotransformation, and cellular context.

Gene expression monitoring is one screening approach that has the potential to serve as a reliable predictor of whole organism effects and specific mechanisms of action of a particular chemical, class of chemicals or mixture of unrelated chemicals. Since changes in gene expression often precede overt morphological and physiological changes, development of molecular endpoints for incorporation into the existing methodology also has the potential to reduce assay duration. To date, changes in morphology of early prometamorphic *X. laevis* tadpoles have been the primary endpoints in the context of a metamorphosis assay (Degitz et al., 2005; Tietge et al., 2005). However, standard toxicological approaches that focus on tissue and organismal

DOI of original article:10.1016/j.aquatox.2007.02.014.
* Corresponding author. Tel.: +1 250 721 6146; fax: +1 250 721 8855.
E-mail address: chelbing@uvic.ca (C.C. Helbing).

0166-445X/$ – see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquatox.2007.02.013

effects fall short in adequately discerning sublethal molecular mechanisms of action.

Metamorphosis involves the transition of the larval tadpole to a juvenile frog. The dramatic structural and functional changes of larval tissues during this developmental process are completely dependent upon thyroid hormones (THs) (Atkinson et al., 1996; Damjanovski et al., 2000; Shi, 2000). As in mammals, thyroxine (T_4) is the main secretory product of the amphibian thyroid gland (Buscaglia et al., 1985) and it is converted by deiodinase activity in peripheral tissues to the more active 3,5,3′-triiodothyronine (T_3) form (Becker et al., 1997; Huang et al., 1999; Kawahara et al., 1999). T_3 functions primarily by regulating gene transcription through high affinity binding to specific nuclear TH receptors (TRs) that interact with T_3-responsive elements (TREs) located within the promoters of target genes (Sap et al., 1986; Weinberger et al., 1986).

Metamorphosis has three distinct developmental phases: premetamorphosis, prometamorphosis, and metamorphic climax. During premetamorphosis, the tadpole is competent to respond to exogenous TH (which can induce precocious metamorphosis) but is functionally athyroid. Prometamorphosis begins with maturation of the thyroid gland and low-level secretion of TH that initiates the first metamorphic changes such as limb growth. TH levels continue to rise and peak dramatically at metamorphic climax, which is characterized by the rapid, overt remodeling of the tadpole. Different tissues respond to TH in different ways. For example, the tail regresses, the brain remodels and the limbs grow and differentiate. The mechanisms whereby tissue response specificity is determined are not fully understood but TR-mediated changes in gene expression play pivotal roles. Therefore, an important aspect in the development of gene expression-based biomarkers is to determine tissue sensitivities to model compounds and to establish the appropriate time point(s) for assessment.

To this end, we exposed early prometamorphic tadpoles to various concentrations of T_3, T_4, and the TH synthesis inhibitors: methimazole (MMI), propylthiouracil (PTU), and perchlorate (PER). MMI and PTU inhibit TH production by blocking the formation of active iodide *via* the peroxidase system (Cooper et al., 1984) while PER ions are competitive inhibitors of iodide uptake (Wolff, 1998). However, all three may have direct effects on peripheral tissues as well. MMI, PTU, and PER have been used to therapeutically treat hyperthyroidism, but PER is also a persistent environmental contaminant (Soldin et al., 2001) and is detected in groundwater at concentrations ranging from 200 μg/L to 3700 mg/L in highly contaminated sites (Urbansky, 1998).

We have previously published the results of our examination of the expression of three gene transcripts, TRα, TRβ, and basic transcription element binding protein (BTEB) in three peripheral tissues: the brain, tail, and hindlimb (Zhang et al., 2006). The brain is a key target organ of TH action in vertebrates where disruption of TH action can lead to permanent developmental defects (Zoeller and Crofton, 2000; Zoeller, 2001; Forrest et al., 2002; Jones and Forrest, 2003; Siesser et al., 2005). The tail and the hindlimb represent two readily-accessible TH-responsive tissues.

We used a combination of cDNA array analyses and quantitative real time polymerase chain reaction (QPCR) to determine the gene expression profile of these tissues to identify gene expression biomarkers for disruption of TH action. This manuscript reports the results found in the brain with a subsequent manuscript describing the results for the tail and hindlimb. Our results indicate that, within 96 h of exposure, the brain is differentially sensitive to the two model hormones T_3 and T_4 and, while MMI, PTU, and PER elicit gene expression responses with several common features, the environmental contaminant, PER, affects several gene transcripts encoding proteins important in neuronal function.

2. Animals, materials and methods

2.1. Experimental animals

X. laevis tadpoles used in this study were obtained from an in-house culture and exposures were done at the US Environmental Protection Agency, Mid-Continent Ecology Division in Duluth. Details about animal husbandry and maintenance were as described previously (Zhang et al., 2006). All protocols were reviewed and approved by the Mid-Continent Ecology Division animal use and care committee.

2.2. Water characteristics

Lake Superior water (LSW) used for all tests was filtered through sand, a 5-μm filter, a 0.45-μm filter, sterilized with ultraviolet light and heated to the appropriate test tank temperature of $20.9 \pm 0.2\,^{\circ}C$ ($n = 588$). Exposure tanks were immersed in a water bath system (water bath temperatures were continuously monitored) to maintain temperature uniformity between tanks. Dissolved oxygen (DO) was measured weekly during all tests using a YSI Model 550A DO meter (Fisher Scientific, Hanover Park, IL) that was calibrated prior to use by the air saturation method. The range of DO measurements across all studies was 6.29–7.80 mg/L. All other water characteristics were measured using methods described by the American Public Health Association (APHA, 1992). The range of pH readings conducted weekly on a minimum of twelve tanks during all tests across all studies was 7.65–8.06. Hardness and alkalinity determinations were made once during each study on a minimum of three tanks including one control and two treatment tanks. The range for total hardness was 47.0–47.5 mg/L $CaCO_3$. The range for all alkalinity measurements was 39.5–40.0 mg/L $CaCO_3$.

2.3. Chemicals

T_4, T_3, methimazole (MMI), 6-propylthiouracil (PTU) and sodium perchlorate (PER) were obtained from Sigma (St. Louis, MO). A stock solution of T_3 was prepared by dissolving 29 mg T_3 into 100 ml of 50 mM NaOH. A diluted stock solution of T_3 (234 nM) was prepared by addition of 10 ml T_3 stock to 19 L of LSW. T_4 stock solutions (5.16 μM) were prepared by dissolving 72.2 mg T_4 in 18 L of LSW. Separate stock solutions for MMI

(1250 mg/L) and PER (492 mg/L) were made in a 19 L glass carboy by dissolving each chemical in LSW using a stir plate and a magnetic stir bar. Stock solutions for PTU (600 mg/L) were made in a 19 L glass carboy using a high speed top stirrer to dissolve the chemical in LSW that had been previously heated to 40 °C.

2.4. Exposure system

A computerized exposure system was used for all studies. This flow-through system, whose components are glass, stainless steel, and Teflon, generated five duplicated exposure concentrations for each chemical with a dilution factor of two for T_4 and T_3, as well as duplicate controls. Exposure tanks were glass aquaria (22.5 cm × 14.0 cm × 16.5 cm deep) equipped with 13 cm standpipes, which resulted in an actual tank volume of 4.0 L. The flow rate to each tank was 25 ml/min. Fluorescent lamps provided a photoperiod of 12 h:12 h light:dark at an intensity that ranged from 61 to 139 lumens at the water surface.

2.5. Animal exposures

2.5.1. Experiment 1 (T_4 and T_3)

Twenty-eight early prometamorphic (NF stage 54; Nieuwkoop and Faber, 1956; Shi, 2000) tadpoles were continuously exposed to three separate T_4 concentrations (10, 20.1, and 40.3 nM), and a LSW control in one exposure set, or five different T_3 concentrations (0.48, 0.97, 1.92, 3.84, and 7.68 nM), and a LSW control in the second exposure set as described in detail in Zhang et al. (2006). Each chemical exposure concentration was replicated twice along with the associated LSW control. At 24, 48 and 96 h two animals per exposure replicate (four animals total per each individual treatment) were randomly selected, euthanized in MS-222, and preserved in RNA*later* (Ambion Inc., Austin, TX, USA) for analysis of gene expression. On exposure day 14, all remaining organisms were euthanized in MS-222, weighed, and developmentally staged in a blind evaluation. Animals exposed to either chemical showed an acceleration of metamorphosis which was published previously (Zhang et al., 2006).

2.5.2. Experiment 2 (MMI, PTU and PER)

NF stage 54 tadpoles were continuously exposed to a single concentration of PTU (20 mg/L), MMI (100 mg/L) or PER (4 mg/L). We have previously shown that exposure to these concentrations resulted in an increase in thyroid gland size at day 8 and significantly delayed metamorphosis at 14 days (Degitz et al., 2005; Tietge et al., 2005). The exposure regimen details are recorded elsewhere (Zhang et al., 2006). Briefly, tadpoles were randomly placed into 24 tanks (20 tadpoles/tank) and exposed (six tanks/chemical) to a single concentration of each chemical. At 24, 48 and 96 h five tadpoles from two of the six tanks (10 tadpoles per each individual treatment) were randomly selected, euthanized in MS-222, and preserved in RNA*later* (Ambion Inc., Austin, TX, USA) for analysis of gene expression.

2.6. Chemical analysis

Water samples collected from all of the exposure sets were measured for the actual concentrations of the chemical additives as described in (Degitz et al., 2005; Tietge et al., 2005). All standard deviations were within 10% of the nominal concentrations. Therefore, the nominal concentrations are reported.

2.7. Isolation of RNA

Brain tissue was collected from each individual tadpole and total RNA was isolated using TRIzol reagent as described by the manufacturer (Invitrogen Canada Inc., Burlington, Ontario, Canada). Mechanical disruption of brain tissue utilized 200 μL TRIzol reagent, a 3 mm diameter tungsten-carbide bead, and safe-lock Eppendorf 1.5 ml microcentrifuge tubes in a Retsch MM301 Mixer Mill (Fisher Scientific Ltd., Ottawa, ON) at 20 Hz for 6 min. Mixing chambers were rotated 180 degrees halfway through the homogenization procedure. Twenty micrograms of glycogen (Roche Diagnostics, Laval, PQ) was added prior to isopropanol precipitation to maximize RNA yield. Isolated RNA was subsequently resuspended in 20 μl diethyl pyrocarbonate (DEPC)-treated RNase-free water and stored at −70 °C.

2.8. Gene expression profiling

Amplified RNA (aRNA) was produced using the MessageAmp aRNA Kit as per the manufacturer's protocol (Ambion) from one microgram brain total RNA isolated from an individual (for the T_3 and T_4 experimental sets) or from three separate pools of total RNA from individuals (for the inhibitor experimental set). This aRNA (500 ng) was annealed with 500 ng random hexamer oligonucleotide (Amersham Biosciences Inc., Baie d'Urfé, QC, Canada), and cDNA was prepared using MMLV RNase H^- Superscript II reverse transcriptase as described by the manufacturer (Invitrogen) with the following modifications: the dNTP mix consisted of 500 μM each of dGTP, dTTP, and dCTP, 4 μM dATP, and 50 μCi [α-^{32}P] dATP (Perkin-Elmer Life Sciences, Inc., Boston, MA, USA). RNA was removed from the radiolabeled cDNA by addition of 10 μL 1M NaOH and incubation at 70 °C for 10 min. Samples were cooled to room temperature and 10 μL of 1M HCl were added to neutralize the reactions. The radiolabeled cDNA was purified using a QIAquick PCR purification kit (Qiagen) and, immediately prior to hybridization, was heat denatured for 5 min at 95 °C and then quickly cooled on ice for 5 min.

The frog MAGEX cDNA array was based upon that described in detail elsewhere (Crump et al., 2002). Briefly, the array consisted of 434 distinct cDNA fragments of ∼500 bp in length. Each fragment was amplified using gene sequence-specific primers and spotted in duplicate at adjacent grid positions on a nylon membrane support. Approximately 90% of the gene sequences present on the array originated from *X. laevis*, while the remainder were isolated from *R. catesbeiana*. Each membrane contained three intron controls to monitor for genomic DNA contamination. Details of the array platform and the raw and normalized data are available from

the Gene Expression Omnibus (Accession numbers GPL4800 and GSE7057; National Center for Biotechnology Information, National Institutes of Health). Prehybridization, hybridization, and posthybridization washes were performed at 65 °C. The radiolabeled cDNA targets from MMI-, PTU-, and PER-exposed animals and their time-matched experimental controls were each divided into three pools of ($n = 3$, 3, and 4) for each treatment time point such that three arrays were probed per treatment and time point. The radiolabeled cDNA targets from T_3 and T_4-exposed animals with their respective time-matched control animals, were derived from three individual animals such that one array was used for each individual. The two highest T_4 concentrations were not analyzed due to the substantial mortality observed during the 14-day T_4 treatment (Zhang et al., 2006).

Hybridizations were carried out in 20 ml of hybridization solution containing 4× SSC, 10% (w/v) dextran sulfate, 1.0% (w/v) SDS, and 0.5% (w/v) Blotto. Prewarmed hybridization solution was added to each hybridization tube (35 mm i.d. × 150 mm length; Amersham) containing the array membrane and prehybridization was allowed to continue for 2 h. Radiolabeled cDNA samples were then added to a final concentration of 5×10^5 cpm/ml and allowed to hybridize overnight. After hybridization, the membranes were rinsed briefly with 50 ml 2× SSC, then washed twice with 50 ml 2× SSC/0.1% SDS for 15 min, once with 50 ml 0.1× SSC/1.0% SDS for 25 min, and rinsed with 50 ml 0.1× SSC. The arrays were placed on 3 MM filter paper (Rose Scientific Ltd., Edmonton, AB, Canada) soaked with ddH_20 and wrapped with plastic wrap. Each processed membrane was exposed to a phosphorimager screen (Molecular Dynamics Inc., Sunnyvale, CA, USA) for 5 days. Hybridization signals were collected using a Storm 820 Gel and Blot Imaging System (Amersham) at 50 μm resolution. The resulting image data were converted to a standard 8-bit TIFF file using Photoshop V5.0 (Adobe Systems Inc., San Jose, CA, USA). Both non-auto- and auto-level images were prepared for analysis in order to account for signal saturation. Non-auto-leveled images provide a linear range of strong signal intensities (such as β-actin) while auto-leveled images allow for analysis of the remaining signals.

Relative expression for each gene target was collected from the image data using ImaGene Version 5.6.1 (BioDiscovery Inc., El Segundo, CA, USA). Signal intensities for each gene and blank position were determined from the median spot pixel intensities and corrected by subtracting the local median background pixel intensities. Signal intensities that were derived from areas of non-specific hybridization on the arrays were not included in the final analysis. A non-signal background was determined from the median intensity value plus one standard deviation of blank positions across the auto-leveled data set, and signal intensities for gene positions exhibiting values below the no signal were adjusted to this value. Saturated gene positions identified in auto-level data were replaced across all data sets by the corresponding values obtained in the non-auto-level analysis.

Data for the three sets were analyzed by time point. Both non-auto- and auto-leveled data for each array were normalized using a geometric mean derived from the median signal intensities from the following genes: ribosomal proteins L8 and S10, GAPDH, ferritin, ubiquitin, NM23/dinucleotide phosphate kinase, cytoplasmic β-actin, and elongation factor-1 α chain. The choice of which transcripts were used for normalization within a time point was dictated by spot quality and consistency (see Section 2.11 below). The gene transcripts used for normalization across a time point are indicated in Supplementary Table 1. After normalization, relative expression values for each gene were determined from the median of signal intensities across the replicate array membranes for each treatment time point. Genes with less than four signal intensity values were not included in further analyses. We considered a two-fold change in signal intensity relative to control treatments as significant in the array experiments based on the detection limitations of the cDNA array analyses.

For hierarchical clustering analysis, data from gene transcripts that were identified to have at least one treatment within a time point that showed a two-fold or greater change relative to the control and which satisfied all data quality measures were imported into Cluster (Eisen et al., 1998) and log transformed. The centered data were then subjected to average linkage clustering to produce a heirarchical cluster tree visualized in Treeview (Eisen et al., 1998).

2.9. Correspondence analysis (CA)

The goal of correspondence analysis here is to represent the association between genes and treatments of the cDNA array experiments in a two-dimensional subspace that maintains most of the information in the data set (Greenacre, 1984, 1993; Fellenberg et al., 2001). Let $\mathbf{N}$ be a matrix with elements n_{ij}, the observation for the ith gene under the jth treatment. The matrix $\mathbf{P}$ contains elements n_{ij}/n, where n is the sum of all n_{ij}. $\mathbf{r}$ and $\mathbf{c}$ represent the sums of the rows and columns of $\mathbf{P}$, respectively, and the standardized matrix $\mathbf{S}$ has elements $s_{ij} = (\mathbf{P}_{ij} - \mathbf{r}_i\mathbf{c}_j)/(\mathbf{r}_i\mathbf{c}_j)^{.5}$. The matrix $\mathbf{S}$ is decomposed into the product of three matrices using singular value decomposition, $\mathbf{S} = \mathbf{U}\mathbf{D}_\mu\mathbf{V}^{\mathrm{T}}$. The columns of $\mathbf{U}$ are the left singular vectors and the columns of $\mathbf{V}$ are the right singular vectors. $\mathbf{D}_\mu$ is a diagonal matrix of singular values of $\mathbf{S}$ with elements vector $\boldsymbol{\mu}$ ordered from largest to smallest. The coordinates of the genes in the new space are given by $\mathbf{F} = \mathrm{diag}(\mathbf{r}^{-.5})\ \mathbf{U}\mathbf{D}_\mu$ The first k (here $k = 2$) columns of $\mathbf{F}$ are the coordinates of the genes in the first k-dimensional subspace. Here we plot column one of $\mathbf{F}$ versus its second column. Lines emanating to the standard coordinates of the treatments given by the first $k = 2$ columns $\boldsymbol{\Gamma} = \mathrm{diag}(\mathbf{c}^{-.5})\mathbf{V}$, allow one to discern associations between the genes and the treatments. The amount of variation in the original data represented in the k-dimensional subspace is proportional to sum of the first k values of $\boldsymbol{\mu}^2$.

Only gene transcripts identified with a two-fold or greater change in at least one time point in any of the experimental treatments were used for CA. Since our goal was to identify potential biomarkers associated with any of the chemical exposures, only gene transcripts which satisfied all data quality measures across all chemical treatments were used for CA. The medians of the combined signal intensity values for each of the chemical treatments were used. For T_3 and T_4 treatments, all five T_3 concentrations and all three T_4 concentrations were combined to

calculate the median values representing each hormone. This was done to ensure that greater weighting was placed on changes in transcript levels that were consistent as a robust biomarker for each condition. Since the data sets were a combination of data generated from non-auto-leveled and auto-leveled data (which were on different scales), we did not directly perform CA on the signal intensity values. Instead, the fold changes relative to the controls were used. In order to preserve appropriate relative weighting of a two-fold (increase) versus 0.5-fold (decrease) change, values ≤ 1.0 were expressed as reciprocals (such that 0.5 became 2, 0.2 became 5, etc.). Thus, a strong increase would be seen as equivalent to a strong decrease and the point representing a given gene transcript would more easily be identified with a particular exposure. Our aim was to identify gene transcripts that had very strong responses that could be easily associated with a particular exposure condition.

2.10. cDNA synthesis and real-time quantitative polymerase chain reaction (QPCR)

Total RNA (0.7 μg) from the brains of individual tadpoles ($n = 4$ for experiment 1 and $n = 10$ for experiment 2) was annealed with 500 ng random hexamer oligonucleotide (Amersham Biosciences Inc., Baie D'urfe, Quebec, Canada) and cDNA was synthesized using Superscript II RNase H^- reverse transcriptase (Invitrogen Canada Inc., Burlington, Ontario, Canada) as described by the manufacturer. The cDNA products were diluted 20-fold prior to PCR amplification.

The expression of individual gene targets was analyzed using a MX4000 real-time quantitative PCR system (Stratagene, La Jolla) and gene-specific primers as shown in Supplementary Table 2. Each 15 μl DNA amplification reaction contained 10 mM Tris–HCl (pH 8.3 at 20 °C), 50 mM KCl, 3 mM $MgCl_2$, 0.01% Tween 20, 0.8% glycerol, 40,000-fold dilution of SYBR Green I (Molecular Probes Inc., Eugene, OR), 200 μM dNTPs, 83.3 nM ROX reference dye (Stratagene), 5 pmol of each primer (Supplementary Table 2), 2 μl of diluted cDNA, and one unit of Platinum Taq DNA polymerase (Invitrogen). The thermocycle program for all gene targets included an initial enzyme activation step at 95 °C (9 min) followed by 40 cycles of 95 °C denaturation (15 s), 55 °C annealing (30 s), and 72 °C elongation (45 s). Controls lacking cDNA template or Taq DNA polymerase were included to determine the specificity of target cDNA amplification. Quadruplicate reactions were performed for each sample and data were averaged and normalized to the expression of the control gene encoding the ribosomal protein L8 using the comparative C_t method (www.dorak.info/genetics/realtime.html). The integrity of amplification reactions was confirmed the presence of a single DNA product following gel electrophoresis and by amplicon sequencing. Gene expression data are presented as fold change relative to control animals within the same treatment period.

2.11. Statistics

Gene expression data sets obtained from three biological replicate arrays for each treatment and time point were analyzed for consistency and absolute agreement using intra-class correlation with a two-way random effects model (SPSS Version 12.0, Chicago, IL, USA). The averages of the intra-class correlation coefficients over the replicate sets was not less than 0.82 with most values greater than 0.9 (Supplementary Table 3). Fold change response to each treatment was determined for each gene relative to the control and genes exhibiting fold change values greater than or equal to two were identified. As an additional quality measure and to reduce the chance of making a type I error, we omitted genes which displayed large variability over the replicate sets. For each gene/replicate set, variability was measured using the equation: (maximum value-minimum value)/2 which is appropriate for samples up to 5 per group (Montgomery, 1991). If the variation estimate for the gene/replicate set was greater than 1.1–1.7 (depending on the data set), the gene was removed from the final list of genes affected following chemical treatment for that time point. If less than four observations were available for a given time point and treatment, that gene was also removed from the final list of genes. In total, 53, 109 and 77 gene transcripts were removed due to this statistical analysis for the TH inhibitor, T_3 and T_4 data sets, respectively, for the 24 h time point. At the 48 h time point, 48, 65, and 95 gene transcripts were removed for the TH inhibitor, T_3 and T_4 data sets, respectively. At the 96 h time point, 50, 46, and 97 gene transcripts were removed for the TH inhibitor, T_3 and T_4 data sets, respectively. The >300 remaining transcripts that showed less than a two-fold change relative to the control were considered as "no change".

Statistical analyses on QPCR data were conducted using SPSS software (SPSS, Chicago, IL, USA). The non-parametric Kruskal–Wallis one-way analysis of variance was conducted within treatment and tissue data sets. Where $P < 0.05$, pairwise comparisons were done using the Mann–Whitney *U*-test. The tank effect in every treatment concentration was not significant ($P > 0.05$) using the Mann–Whitney *U*-test, therefore, the data of two replicate tanks were combined for further statistical analyses. Steady-state mRNA levels of the testing gene in a given exposure time and chemical concentration/treatment was compared with the respective controls using the Mann–Whitney *U*-test.

3. Results

3.1. cDNA array analyses of gene expression

We first evaluated the overall number of genes affected by each of the treatments as a function of time. At any given time point, the number of gene transcripts identified upon exposure to MMI, PTU, or PER was comparable between the three chemical treatments (Table 1). At 24 and 96 h, there were approximately 40 transcripts identified which was reduced at 48 h to less than half (Table 1). In contrast, T_3 exposure initially resulted in 66 affected transcripts whose number decreased dramatically by 96 h (Table 1) suggesting that the response to this hormone is relatively rapid. In contrast, the number of gene transcripts affected by T_4 exposure did not reach maximal levels until 96 h which may be more in line with a conversion step to the more active

Table 1
Number of gene transcripts identified as ≥2-fold in the cDNA array experiments

Time point (h)	Treatment				
	MMI	PTU	PER	T_3	T_4
24	42	39	40	66	34
48	19	19	14	24	36
96	35	39	38	9	52

T_3 form (Table 1). Greater detail regarding the gene transcript identities for each exposure time point and their fold change are listed in the Supplemental Data.

The identified genes were grouped into eleven functional categories and the frequencies of occurrence per category were plotted in Fig. 1. Gene transcripts encoding proteins involved in transcription and cell growth control were the most frequently represented at 24 h of T_3 treatment which was largely maintained at 48 h, but was dramatically decreased by 96 h. T_4 treatment also resulted in the highest number of gene transcripts encoding proteins controlling transcription at 24 h. However, the similarity to T_3 was not maintained at later time points. In addition to affecting transcripts important in transcription, gene transcripts encoding proteins involved in signal transduction, structural, and apoptosis/protein processing showed more representation at 48 h. At 96 h, gene transcripts encoding proteins involved in transcription and hormonal regulation were most represented.

MMI, PTU, or PER exposure regardless of time point produced strikingly similar graphical profiles. Exposure to these chemicals for 24 h resulted in a more even distribution of gene transcripts amongst all of the categories with the highest representation encoding proteins important in transcription, signal transduction, metabolism/biosynthesis, and transport/binding. MMI affected transcripts encoding structural proteins to a greater extent than PTU or PER. Representation in virtually all of these categories was greatly reduced at 48 h. Transcripts encoding proteins involved in transcription, hormonal regulation, apoptosis/protein processing, and structural proteins were most represented at 96 h. Taken together, these data indicate that, although the THs showed similarity in affecting transcripts in the transcription category the most, distinct expression profiles were evident suggesting differential effects of T_3 and T_4 on the brain. In contrast, the greater degree of similarity in

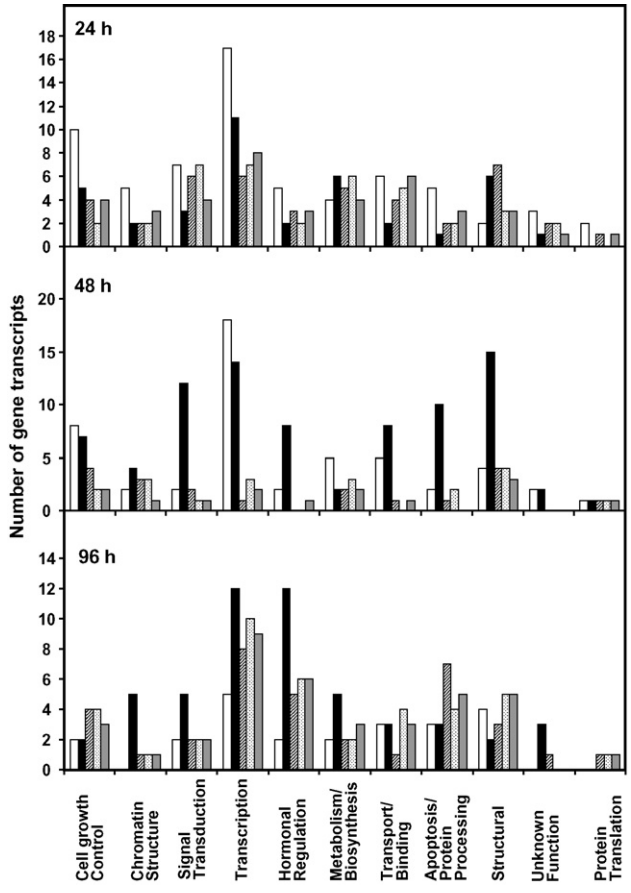

Fig. 1. Distribution of observed transcripts by functional category. The number of gene transcripts identified as having ≥2-fold change compared to the control detected within each functional group using array analysis is shown for T_3 (white bar), T_4 (black bar), MMI (hatched bar), PTU (stippled bar), and PER (grey bar) at the indicated time points. The functional codes are indicated on the *X*-axis.

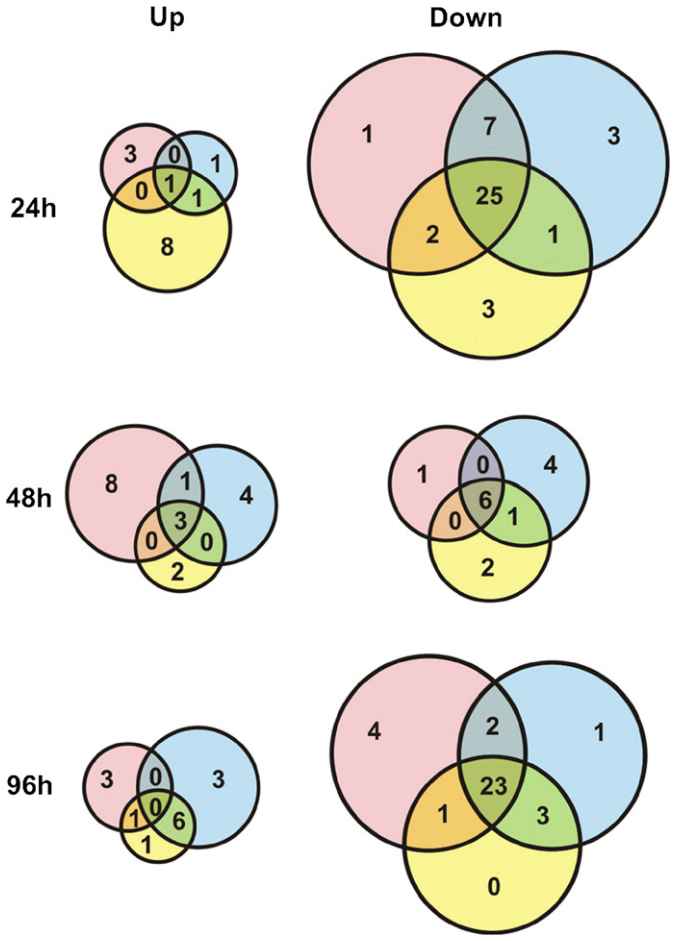

Fig. 2. Overlap of brain gene expression responses for MMI, PTU, and PER. Venn diagrams depict the extent of overlap in gene transcript levels that are increased (up) or decreased (down) following exposure to MMI (red), PTU (blue), and PER (yellow) at the indicated time points. The number of gene transcripts identified is indicated in each field with the relative size of the circles indicating the total number of gene transcripts affected per treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expression profiles according to functional category between the three TH synthesis inhibitors suggests a common mode of action.

We next examined the extent of overlap of the effects of MMI, PTU, and PER on gene transcript levels in relational Venn diagrams (Fig. 2). At 24 and 96 h, the total number of gene transcripts whose steady-state levels decreased greatly outnumbered the gene transcripts whose levels increased (Fig. 2). In contrast, the total number of increased and decreased gene transcripts was equivalent at 48 h. The majority of transcripts that

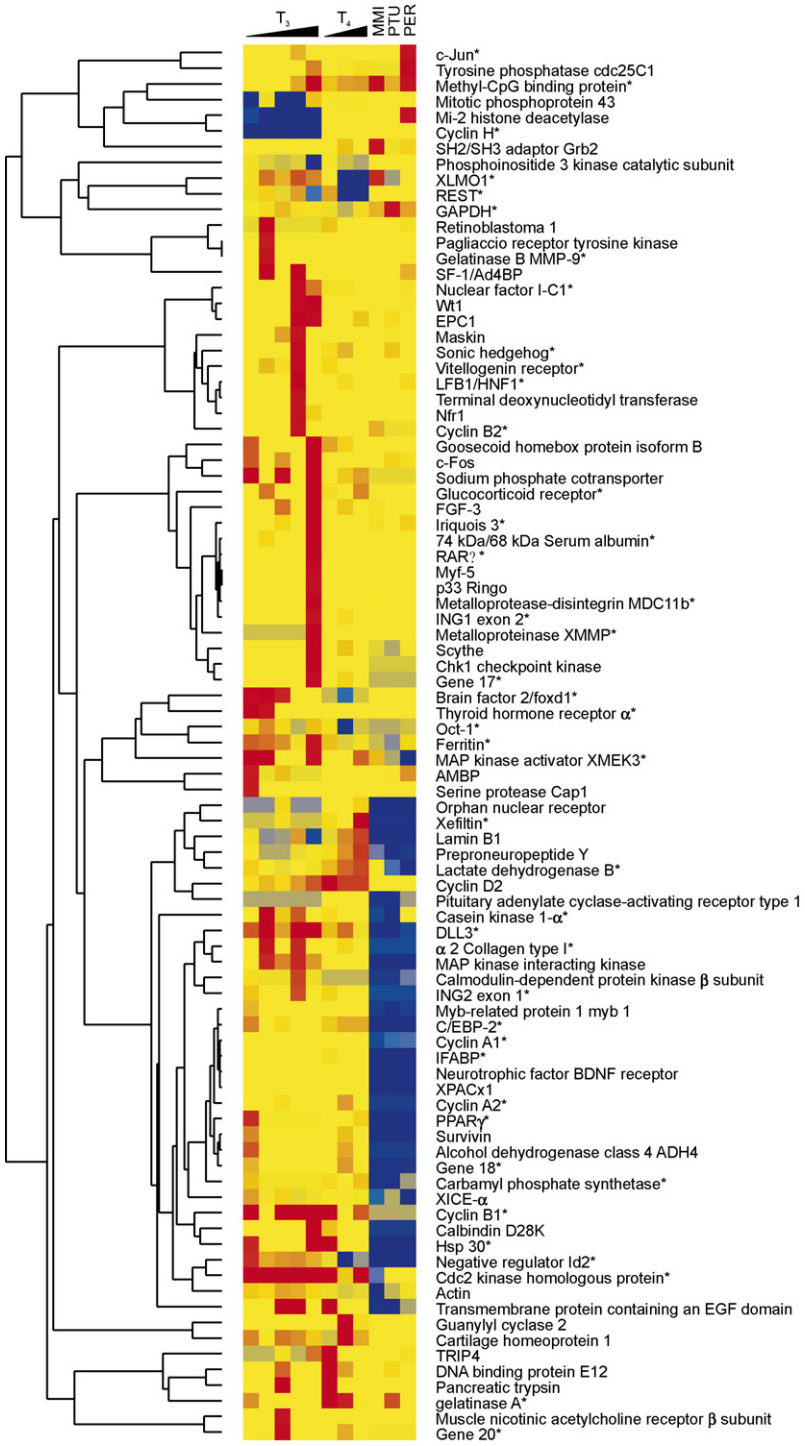

Fig. 3. Agglomerative hierarchical clustering analysis of gene expression in the brain of *Xenopus laevis* at 24 h exposure as visualized by an hierarchical tree. An increase in relative gene expression is depicted in red, whereas a decrease is depicted in blue. No change is indicated in yellow. The gene identities are indicated to the right. The asterisks denote gene transcripts identified as T_3-responsive in the tadpole brain or other tissues (Shi and Hayes, 1994; Denver et al., 1997; Sachs et al., 2001; Crump et al., 2002; Helbing et al., 2003; Krain and Denver, 2004; Wagner and Helbing, 2005; Das et al., 2006; Veldhoen et al., 2006a,b,c). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

decreased were common to all three chemicals whereas this was not the case with the transcripts with increased levels. Despite the early indication at 24 h that MMI and PTU had more affected gene transcripts in common than either chemical with PER, this feature was not maintained at later time points. In fact, at 96 h, PTU and PER had more gene transcripts in common than either chemical did with MMI (Fig. 2).

We then performed an agglomerative hierarchical clustering analyses (Eisen et al., 1998) on the data for each time point that passed all of the quality and consistency criteria for all chemical treatments. A small cluster of gene transcripts were decreased by T_3 at all concentrations tested, but not upon T_4 or inhibitor exposure. This cluster included Mi-2 histone deacetylase and cyclin H, both which are involved in transcriptional regulation. There were surprisingly few gene transcripts that were affected by all concentrations tested. In fact, several gene transcripts only responded to the higher concentrations (3.84 and 7.68 nM) of T_3 (Fig. 3). We identified a major cluster of gene transcripts at the 24 h time point (Fig. 3) characterized by decreased levels upon exposure to MMI, PTU, and PER relative to matched controls. With very few exceptions, these three chemicals affected gene expression in a similar way. Such exceptions included increased c-Jun and tyrosine phosphatase cdc25C1 transcript levels upon PER treatment, increased levels of SH2/SH3 adaptor grb2 and cdc2 kinase homologous protein transcripts upon MMI exposure, and increased gelatinase A transcripts upon exposure to PTU (Fig. 3).

By 48 h, the number of genes showing any response to T_3 was greatly reduced and primarily was represented by treatment at the second highest concentration tested (3.84 nM; Fig. 4). A marked appearance of gene transcripts whose levels decreased upon T_4 exposure compared to matched controls was apparent (Fig. 4). A smaller number of transcripts increased upon T_4 exposure, mainly at the middle concentration examined (Fig. 4). The cluster representing gene transcript levels that were decreased upon MMI/PTU/PER exposure was diminished in size at this time point (Fig. 4) whereas transcripts that were increased were more prominent (Fig. 4) compared to the 24 h time point (Fig. 3). As at 24 h, several gene transcripts showed a common response among the three TH synthesis inhibitors. However, greater prominence in the number of gene transcripts that were affected by MMI only was evident relative to the 24 h time point (Fig. 4).

Finally by 96 h, any substantial cluster reflective of a T_3-related exposure was not observed (Fig. 5) whereas separate clusters of genes representing decreased gene transcript levels

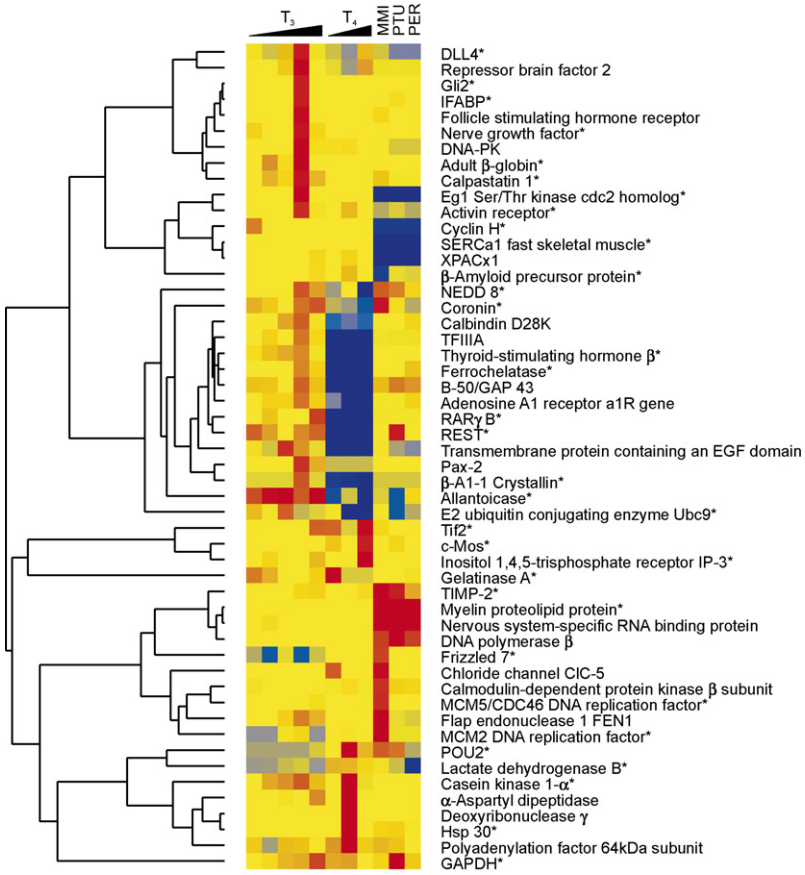

Fig. 4. Agglomerative hierarchical clustering analysis of gene expression in the brain of *Xenopus laevis* at 48 h exposure as visualized by an hierarchical tree. An increase in relative gene expression is depicted in red, whereas a decrease is depicted in blue. No change is indicated in yellow. The gene identities are indicated to the right. The asterisks denote gene transcripts identified as T_3-responsive in the tadpole brain or other tissues (Shi and Hayes, 1994; Denver et al., 1997; Sachs et al., 2001; Crump et al., 2002; Helbing et al., 2003; Krain and Denver, 2004; Wagner and Helbing, 2005; Das et al., 2006; Veldhoen et al., 2006a,b,c). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

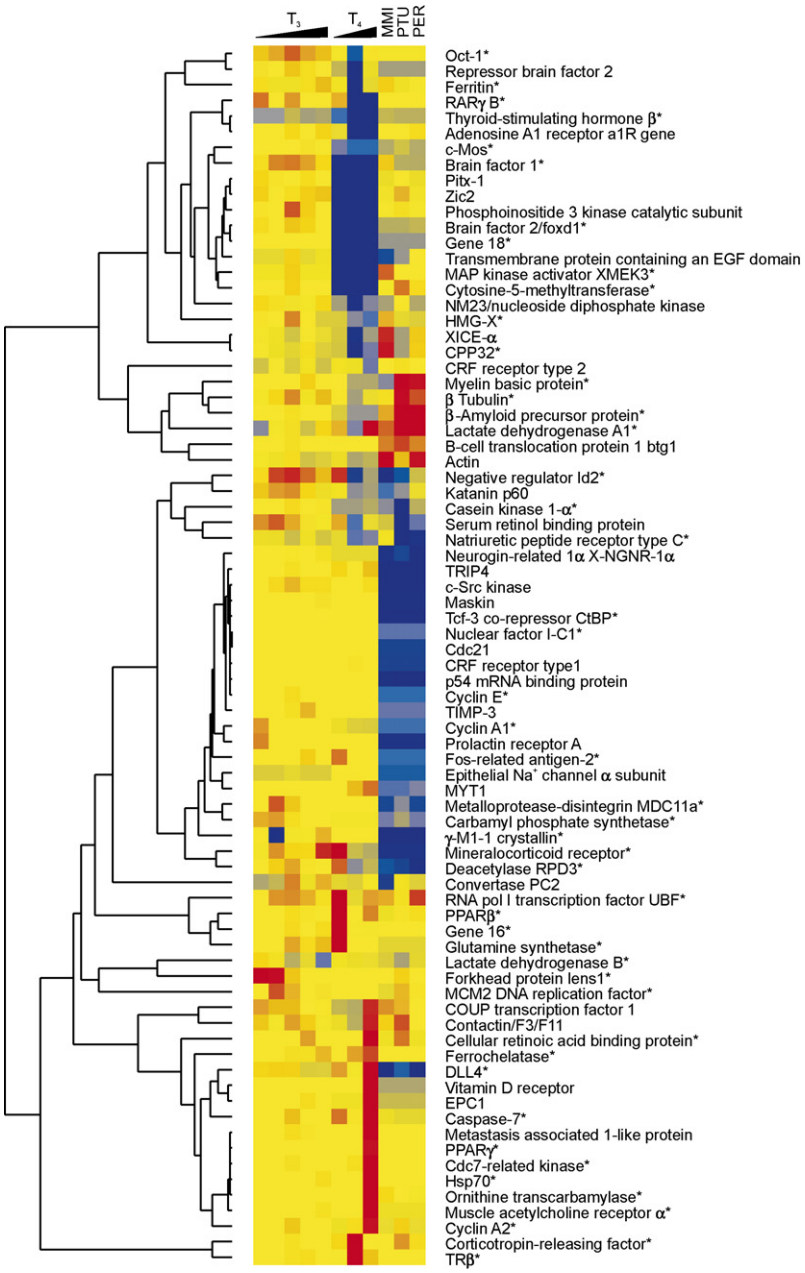

Fig. 5. Agglomerative hierarchical clustering analysis of gene expression in the brain of *Xenopus laevis* at 96 h exposure as visualized by an hierarchical tree. An increase in relative gene expression is depicted in red, whereas a decrease is depicted in blue. No change is indicated in yellow. The gene identities are indicated to the right. Three major clusters are observed at this time point. The asterisks denote gene transcripts identified as T_3-responsive in the tadpole brain or other tissues (Shi and Hayes, 1994; Denver et al., 1997; Sachs et al., 2001; Crump et al., 2002; Helbing et al., 2003; Krain and Denver, 2004; Wagner and Helbing, 2005; Das et al., 2006; Veldhoen et al., 2006a,b,c). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

relative to matched controls for T_4, MMI, PTU, and PER were readily detected (Fig. 5). Again, gene transcript level changes due to MMI, PTU, and PER exposures tended to cluster together (Fig. 5).

The heirarchical clustering gave good indications of general strengths of gene expression responses to the test chemicals relative to the controls. However, it was difficult to assess from this analysis which gene transcripts were the best candidates for gene expression biomarkers that could be used as indicators for exposure to TH agonists or antagonists. A particularly important attribute for a gene expression biomarker is sensitivity at multiple concentrations (e.g. for T_3 and T_4) or as being representative of a particular class (such as TH antagonist). In order to better determine these characteristics, we calculated the median responses across all concentrations (for T_3 or T_4) or different types of inhibitors (MMI, PTU, and PER) and used these values to generate correspondence analysis (CA) plots. CA is a statistical visualization method for presenting the associations between the two dimensions of a data matrix. It displays the rows and columns of a two-way table as points in a low-dimensional graph, such that the positions of the row and column points are consistent with their associations in the data matrix. CA graphs

can assist in detecting relationships among the variables of a data matrix because they can reveal relationships that would not be detected in a series of pair-wise comparisons of variables. The only data requirement is a rectangular data matrix with non-negative entries. Traditionally, correspondence analysis has been used to display categorical data from the social sciences (Greenacre, 1984, 1993; Blasius and Greenacre, 1998), but recently its application has been extended to microarray data (Fellenberg et al., 2001; Culhane et al., 2002; Tan et al., 2004; Busold et al., 2005).

The same data sets used to generate the heirarchical clusters were subjected to CA to generate the two-dimensional plots in Fig. 6. Combining the first (*X*-axis) and second (*Y*-axis) dimensions accounts for 83, 86, and 90% of the data variation for the 24, 48 and 96 h time points, respectively. The further away a given point representing a gene transcript is relative to the origin on a CA plot, the more the observed response deviates from an average response. If the point lies close to or along the line representing a treatment condition, then the more strongly related the gene transcript response is to that exposure. The CA coordinates for each exposure condition are represented by the different colored lines in Fig. 6. If CA coordinates are located in the same half of the CA plot, those conditions are more related. This is particularly true for the halves represented by the *X*-axis which accounts for >65% of the data variation.

At all time points, the CA coordinates for MMI, PTU, and PER exposures colocalized to the same half of the CA plot. At the 24 h time point, T_3 and T_4 exposures also colocalized to the other half of the CA plot. At later time points, T_3 exposure was not associated strongly with any other exposure condition (Fig. 6).

We identified several groups of gene transcripts that associated with a given exposure or group of exposure conditions. The identities of each of the gene transcripts indicated in the ovals in Fig. 6 are presented in Supplementary Table 4. At 96 h, more gene transcripts were detected as having a stronger association with T_4 or MMI/PTU/PER exposure (Fig. 6B and C). Several gene transcripts were associated with MMI or PTU/PER exposure at this time point (Fig. 6C). Of particular note is the very distinctive clustering of gene transcripts along the line representing T_4 exposure at 96 h (Fig. 6C). At these two later time points, there were no gene transcripts that displayed strong association with T_3 exposure.

3.2. Identification of gene expression biomarkers using QPCR

For a gene expression biomarker, establishment of the timing of sampling is important to reliably determine statistically significant increases or decreases in transcript levels upon exposure to a particular chemical or chemical class. Of equal importance is the determination of no significant change upon exposure to other chemicals. The CA plots identified potential biomarker candidates and indicated that additional gene transcripts encoding proteins important in neuronal function and TH signalling would be excellent additional candidates (Supplementary Table 1). We developed QPCR methods for a selection

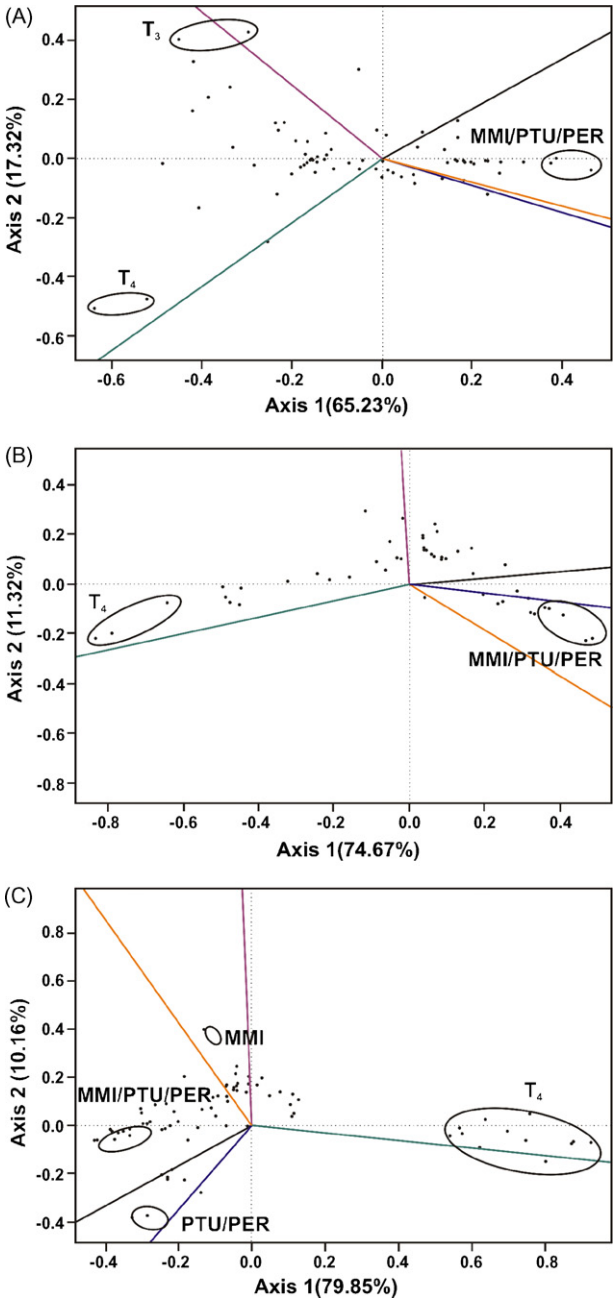

Fig. 6. Correspondence analysis plots for (A) 24 h, (B) 48 h, and (C) 96 h time points. The CA coordinates for genes are displayed as dots and the treatment axes as coloured lines (orange, MMI; blue, PTU; black, PER; brown, T_3; green, T_4). Genes that tend to be differentially expressed in one given treatment will tend to gravitate towards the coordinate for that treatment. Genes that are differentially expressed in more than one treatment lie midway between the respective treatments. The plot also depicts the orientation of the treatments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of these gene transcripts using primers listed in Supplementary Table 2 and the resultant bar graphs are shown in Fig. 7. As our initial experimental design kept all RNA from individual animals separate, we were able to determine transcript levels directly from a greater number of biological replicates using unamplified RNA as source material (in contrast to the aRNA that was used for the array analysis).

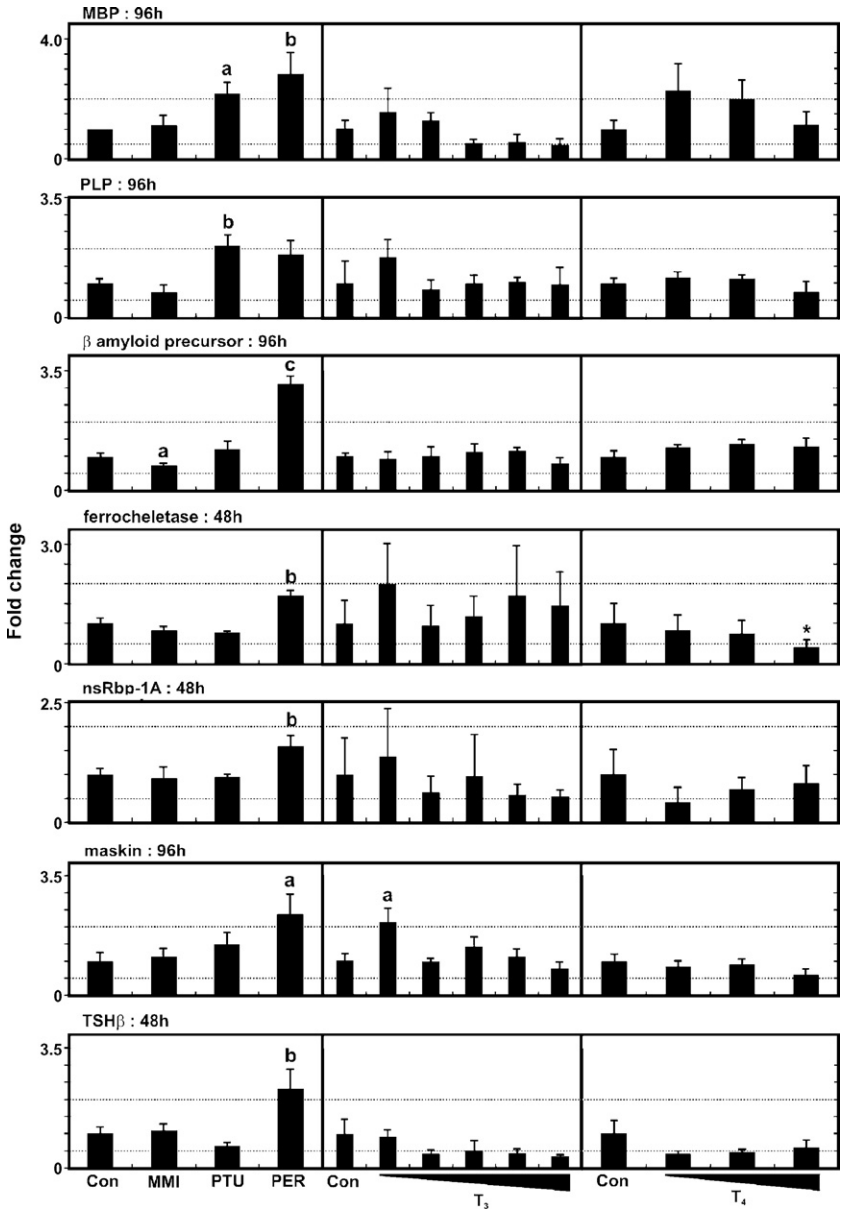

Fig. 7. QPCR analyses of gene expression biomarker candidates in the brain of *Xenopus laevis*. The levels of mRNA transcripts encoding β amyloid precursor protein, myelin proteolipid protein (PLP), myelin basic protein (MBP), ferrochelatase, nervous system-specific RNA binding protein (nsRbp), maskin, and thyroid stimulating hormone β (TSHβ) at the indicated time points were determined (bars). Expression was normalized relative to ribosomal protein L8 transcript levels and fold changes were calculated relative to time-matched vehicle controls. Error bars represent SEM and those treatments showing a significant change in mRNA levels relative to the controls are indicated by "a" ($P < 0.05$), "b" ($P < 0.01$), or "c" ($P < 0.001$). The asterisk indicates that at higher concentrations (80.6 and 161.2 nM, a further significant reduction in transcript levels were observed (data not shown). Although not significant at 96 h, PLP transcript levels are significantly increased in the PER exposure at 48 h (data not shown). The results are the average of 8–10 biological replicates for each condition in the inhibitors set and four biological replicates for each concentration tested in the T_3 and T_4 sets. Horizontal stippled lines denote two-fold decrease and increase in mRNA levels compared to the controls.

Two transcripts that encode proteins important in myelination, myelin basic protein and myelin proteolipid protein, showed increased levels of transcripts upon PTU and PER exposure but not upon MMI exposure (Fig. 7). Exposure to either T_3 or T_4 did not result in a significant change in transcript levels. A transcript encoding the β amyloid precursor protein was also increased substantially upon PER exposure whereas MMI exposure resulted in a significant decrease (Fig. 7). Neither PTU, T_3, or T_4 exposure affected the steady-state levels of this transcript. PER exposure also resulted in a significant increase in ferrochelatase, neuron-specific RNA binding protein-1A (nsRbp-1A), maskin, and TSHβ mRNAs (Fig. 7). Of these, nsRbp-1A transcripts were not affected by T_3 or T_4 exposure. Maskin was also largely unaffected with the exception of the lowest T_3 concentration tested which showed a significant increase in this transcript (Fig. 7). It should be noted that cDNA array and QPCR approaches are not necessarily equivalent since they have differential potential for detecting related transcript

isoforms, many of which have not yet been fully characterized. This is likely the case for nsRBp-1A and maskin (compare Fig. 7 to Supplemental Data) as at least three nsRBp-related sequences (Genbank accession nos. M34894-5 and BC088603) and two highly similar maskin-type sequences (Genbank accession nos. AF200212 and BC075505) are known in *Xenopus*.

In the case of ferrochelatase, increasing concentrations of T_4 reduced the steady state levels of this transcript whereas T_3 showed no significant change albeit there was a higher degree of variation in this treatment set for this transcript (Fig. 7). TSHβ mRNA levels showed a consistent decrease upon T_4 and T_3 exposure. However, none of the data reached statistical significance at the concentrations tested (Fig. 7).

4. Discussion

Given the absolute requirement of TH for proper brain functioning and its particular sensitivity to perturbation (Turque et al., 2005; Zoeller, 2005; Helbing et al., 2006, 2007; Veldhoen et al., 2006a,b,c; Veldhoen et al., 2006a,b,c), identification of gene expression targets is important to assess sublethal toxicity end points that could impact brain development and function. Using a combination of cDNA array and QPCR analyses, we identified the expression profiles in the brains of tadpoles exposed to T_3, T_4, MMI, PTU, and PER. All concentrations of chemicals tested showed clear acceleration or inhibition of metamorphic parameters within the context of a 14-day metamorphosis assay (Degitz et al., 2005; Tietge et al., 2005; Zhang et al., 2006). In keeping with previous indications (Zhang et al., 2006), the gene expression profiles generated at three time points (24, 48, and 96 h) showed a particular sensitivity of the brain to T_4, MMI, PTU, and PER with little sensitivity to T_3.

Previous work from other laboratories have identified several gene transcripts whose expression levels change in response to T_3 treatment at concentrations ~10 times higher than the highest concentration used in this study (Denver et al., 1997; Das et al., 2006). The most recent study pretreated NF stage 54 tadpoles with 1 μM MMI for 1 week prior to exposure to 100 nM T_3 (Das et al., 2006). No studies on brain gene expression profiles have been reported in tadpoles with the lower T_3 concentrations used in this study or with T_4. We found that, although the animals clearly responded to T_3 treatment primarily by an increase in gene transcript levels over a short time frame (within 48 h; Figs. 3 and 4), most of the genes previously identified as being T_3-responsive in the brain with higher T_3 concentrations were not identified in this study. However, a majority of gene transcripts identified as responsive to T_3 in the brain have been described as being T_3-responsive in other tissues (Figs. 3–5).

Of note is that gene transcripts that were observed to be strongly decreased in T_3-responsive tissues using concentrations higher than used in this study (Das et al., 2006) showed no change (genes 16 and 18; Fig. 5) or increased (genes 17 and 20; Fig. 3) in response to this hormone. Gene 16 was increased upon exposure to the lowest T_4 concentration tested (Fig. 5) while gene 18 was decreased at all T_4 concentrations (Fig. 5). Only gene 17 and 18 transcripts were decreased upon exposure to MMI, PTU, or PER (Figs. 3 and 5). The example of this set of down-regulated genes serves to illustrate that the brain's TH-dependent response varies according to the concentration of the exposure chemical. Typical of a hormonal response, high concentrations of hormone served to quench its own signal in an auto-feedback loop. Low concentration effects are more likely to be observed in the context of endocrine disruption and are, therefore, very important to identify.

Comparison of the responses observed to T_3 versus T_4 reveals that there is very little overlap in the profiles of these two THs. In fact, several gene transcripts show opposite responses (e.g. increased in T_3, decreased in T_4) that are not predicted by a simple conversion of T_4 to T_3 in the cell and correcting for differences in binding affinities to the TRs (Zhang et al., 2006). A similar observation was made with the expression levels of TRβ and BTEB transcripts (Zhang et al., 2006). Despite this, about 60% of the responsive gene transcripts identified in the T_4 exposures have previously been identified as T_3-responsive in tadpole tissues usually using higher concentrations of T_3 (Figs. 3–5). It is possible that multiple factors are at play to explain the observed patterns. Conversion of T_4 to T_3 by deiodinases could require a temporal delay in expression that may not have been detected at the time points tested. However, it is unlikely that this plays a major role since neither T_4 nor T_3 affected deiodinase II or III transcript levels at the concentrations or time points tested (data not shown). Recent work indicates that these hormones have a differential ability to induce phosphorylation signaling cascades that influence hormone-inducible gene expression (reviewed in Bassett et al., 2003), and this may account for the differences in influence of T_3 and T_4 on gene expression in the brain. However, this remains to be determined.

TH can modulate the transcriptome within hours of exposure by altering TR activity on TH-responsive gene promoters. In contrast, the impact of MMI, PTU, and PER has largely been attributed to an inhibition of TH synthesis by the thyroid gland which may take days for the impact to be felt on peripheral tissues. We observed surprisingly similar, rapid (within 24 h) responses of transcripts to the three goitrogens even though these three chemicals inhibit TH synthesis and release through different mechanisms. We currently cannot clearly distinguish between TH synthesis inhibition effects and direct tissue effects and it is possible that these chemicals may target similar brain regions, such as the hypothalamus or pituitary, as part of a feedback mechanism. However, more detailed analyses of specific brain regions are required to determine if this idea is supported.

Despite the common features shared between responses to MMI, PTU, and PER within a time point, there was very little resemblance in expression profiles of each chemical *between* time points. This accentuates the strong temporal component to changes in transcript expression levels that needs to be kept in mind. However, clear distinctions could be made between the different chemicals within a time point as well but there were no clear-cut subgroupings between modes of action on TH synthesis, for example, between MMI/PTU and PER. In fact, PTU tended to group with PER particularly at the latest time point tested. It is possible that the reason for this observation lies in the direct effects of these chemicals upon the brain tissue rather than the secondary effect of altering TH synthesis over this shorter

Table 2
Gene names of groups identified in CA plots

Gene transcript identity	Treatment association	Time point (h)
Cyclin H[a,b]	T_3	24
Mi-2 histone deacetylase		
REST corepressor[a,b]	T_4	24
XLMO1 neuronal-specific transcription factor[b]		
CCAAT/enhancer core binding protein C/EBP-2[a,b]	MMI/PTU/PER	24
Neurotrophic factor BDNF receptor		
Xeroderma pigmentosum group A complementing protein XPACx1		
Ferrochelatase[b]	T_4	48
Retinoic acid receptor RARγ B[b]		
Thyroid stimulating hormone β[c]		
Eg1 kinase (cdc2 homolog)[a,b]	MMI/PTU/PER	48
Myelin proteolipid protein[b]		
Nervous system-specific RNA binding protein		
SERCa1 fast skeletal muscle[b]		
Xeroderma pigmentosum group A complementing protein XPACx1		
Adenosine A1 receptor (a1R gene)	T_4	96
Brain factor 1[b]		
Brain factor 2		
Cytosine-5-methyltransferase[a,b]		
Gene 18[b]		
Mitogen associated protein kinase activator (XMEK3)[b]		
Phosphoinositide 3 kinase catalytic subunit		
Pitx-1 homeodomain transcription factor		
Retinoic acid receptor RARγ B[b]		
Thyroid-stimulating hormone β[c]		
Transmembrane protein containing an EGF domain		
Zinc finger DNA binding protein zic2		
Argininosuccinate lyase	MMI/PTU/PER	96
Cdc21		
c-Mos		
Corticotropin-releasing factor receptor type1		
c-Src kinase		
γ-M1-1 crystallin[a]		
Deacetylase (RPD3)[b]		
Distal-less 4 (DLL4)[b]		
E2 ubiquitin conjugating enzyme (Ubc9)[b]		
Epithelial Na^+ channel α subunit		
Gelatinase A[b]		
Maskin		
Mineralocorticoid receptor[a,b]		
Neurogin-related 1α (X-NGNR-1α)		
p54 mRNA binding protein		
Prolactin receptor A		
Tcf-3 co-repressor CtBP[b]		
Thyroid receptor interacting protein TRIP4		
β-amyloid precursor protein[b]	PTU/PER	96
Lactate dehydrogenase A1[a,b]		
Convertase PC2	MMI	96

[a] Identified as T_3-responsive in the tadpole brain (Das et al., 2006; Denver et al., 1997; Krain and Denver, 2004). Note that all of these studies used higher concentrations of T_3 and Das et al. (2006) pretreated tadpoles with 1 μM MMI for 1 week prior to treatment with 100 nM T_3.

[b] Identified as T_3-responsive in tadpole tissues other than the brain (Crump et al., 2002; Das et al., 2006; Helbing et al., 2003; Krain and Denver, 2004; Sachs et al., 2001; Shi and Hayes, 1994; Veldhoen et al., 2006a,b,c; Wagner and Helbing, 2005).

[c] Increased upon ethenylthiourea treatment (Opitz et al., 2006).

time frame. Nevertheless, several key gene transcripts known to play important roles in neural development and function were targets of (primarily) PER. To our knowledge, this is the first reported indication of the perturbation of gene expression in the brain by PER suggesting that this environmental contaminant has the potential to disrupt normal signaling in the brain over a short time frame. This is certainly an area of ongoing concern as PER is a common contaminant in drinking water. However, a clear linkage between PER contamination in drinking water and attention deficit disorders, autism, and lowered intelligence has yet to be determined (Bekkedal et al., 2004).

Sixty percent of the gene transcripts identified within 48 h of exposure to MMI, PTU, and/or PER have previously been associated with a T_3-induced response in tadpole tissues (Figs. 3 and 4). At 96 h, this number drops to ∼40% which may indicate a transient compensatory increase in endogenous TH levels. In some cases, such as that observed for ferrochelatase and TSHβ transcripts (Fig. 7), opposite gene expression profiles were observed when comparing the THs to MMI/PTU/PER. The use of these gene transcripts as biomarkers might be useful. However, neither of these two transcripts reached a statistically significant decrease in our experiments. A higher number of animals for gene expression analysis may be needed to be able to distinguish significant effects.

The cDNA array analyses revealed several strong candidate biomarkers for exposure to T_4 and MMI/PTU/PER, which contrasts with the paucity of T_3 biomarker candidates. We characterized some of these transcripts using QPCR, but further development of the remaining candidates is warranted especially to more accurately capture population variation. Candidate genes were identified at all three time points with the most strongly exposure-related transcripts appearing at the later time points as revealed by correspondence analyses (Table 2).

In conclusion, through an extensive comparison of gene expression profiles in the brain generated upon exposure to T_3, T_4, MMI, PTU, and PER, we have been able to identify several potential biomarkers that could be developed further for incorporation into a metamorphosis-based screening assay. The brain is particularly sensitive to gene expression alteration by MMI, PTU, PER, and T_4. The data reiterate the importance of selecting the appropriate sampling time point and tissue. For the brain, 48 and 96 h gave the most reliable results with the greatest number of biomarker candidates. From these results and those presented elsewhere (Helbing et al., 2006, 2007; Zhang et al., 2006), additional candidate gene biomarkers from other tissues will enhance the confidence in the assignment of chemical exposures. Therefore, measurement of this tissue's response with that of others which respond readily to T_3 (such as tail) will greatly increase the power of the metamorphic assay.

Acknowledgements

We thank Jeff Ricketson for technical assistance. This work was funded by an EPA cooperative agreement and, in part by the EJLB Foundation, to CCH. CCH is a Michael Smith Foundation for Health Research scholar and is recipient of a NSERC University Faculty Award and an early career award

for applied ecological research from the Society of Environmental Toxicology and Chemistry. This work was supported in part with funding provided by the American Chemistry Council through a Cooperative Research and Development Agreement (CRADA) with the EPA's Mid Continent Ecology Division. The information in this document has been funded in part by the US Environmental Protection Agency. It has been subjected to review by the National Health and Environmental Effects Research Laboratory and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2007.02.013.

References

APHA, 1992. Standard methods for the examination of water and wastewater. DC American Public Health Association, American Water Works Association, Water Environment Foundation, Washington.

Atkinson, B.G., Helbing, C., Chen, Y., 1996. Reprogramming of gene expression during amphibian metamorphosis. In: Gilbert, L.I., Tata, J., Atkinson, B.G. (Eds.), Metamorphosis: postembryonic reprogramming of gene expression in amphibian and insect cells. Academic Press, San Diego, pp. 539–566.

Bassett, J., Harvey, C.B., Williams, G.R., 2003. Mechanisms of thyroid hormone receptor-specific nuclear and extra nuclear actions. Mol. Cell. Endocrinol. 213, 1–11.

Becker, K.B., Stephens, K.C., Davey, J.C., Schneider, M.J., Galton, V.A., 1997. The type 2 and type 3 iodothyronine deiodinases play important roles in coordinating development in *Rana catesbeiana* tadpoles. Endocrinology 138, 2989–2997.

Bekkedal, M., Arfsten, D., Mattie, D., 2004. An evaluation of neurobehavioral tests used to assess the neurodevelopmental effects of early ammonium perchlorate exposure. J. Tox. Environ. Health A 67, 835–844.

Blasius, J., Greenacre, M., 1998. Visualization of categorical data. Academic Press, London.

Buscaglia, M., Leloup, J., deLuze, A., 1985. In: Balls, M., Bownes, M. (Eds.), Metamorphosis. Clarendon, New York, pp. 273–293.

Busold, C., Winter, S., Hauser, N., Bauer, A., Dippon, J., Hoheisel, J., Fellenberg, K., 2005. Integration of GO annotations in correspondence analysis: facilitating the interpretation of microarray data. Bioinformatics 21, 2424–2429.

Cooper, D.S., Kieffer, J.D., Saxe, V., Mover, H., Maloof, F., Ridgway, E.C., 1984. Methimazole pharmacology in the rat: studies using a newly developed radioimmunoassay for methimazole. Endocrinology 114, 786–793.

Crump, D., Werry, K., Veldhoen, N., van Aggelen, G., Helbing, C.C., 2002. Exposure to the herbicide acetochlor results in increased thyroid hormone-dependent gene expression and accelerated metamorphosis in *Xenopus laevis*. Enivron. Health Persp. 110, 1199–1205.

Culhane, A.G.P., Considine, E., Cotter, T., Higgins, D.G., 2002. Between-group analysis of microarray data. Bioinformatics 18, 1600–1608.

Damjanovski, S., Amano, T., Li, Q., Ueda, S., Shi, Y.-B., Ishizuya-Oka, A., 2000. Role of ECM remodeling in thyroid hormone-dependent apoptosis during anuran metamorphosis. Ann. New York Acad. Sci. 926, 180–191.

Das, B., Cai, L., Carter, M., Piao, Y.-L., Sharov, A., Ko, M., Brown, D.D., 2006. Gene expression changes at metamorphosis induced by thyroid hormone in *Xenopus laevis*. Devel. Biol. 29, 342–355.

Degitz, S.J., Holcombe, G.W., Flynn, K.M., Kosian, P.A., Korte, J.J., Tietge, J.E., 2005. Progress towards development of an amphibian-based thyroid screening assay using *Xenopus laevis*. Organismal and thyroidal responses to model compounds 6-propylthiouracil, methimazole, and thyroxine. Tox. Sci. 87, 353–364.

Denver, R.J., Pavhi, S., Shi, Y.B., 1997. Thyroid hormone-dependent gene expression program for *Xenopus* neural development. J. Biol. Chem. 272, 8179–8188.

Eisen, M.B., Spellman, P.T., Brown, P.O., Botstein, D., 1998. Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. U.S.A. 95, 14863–14868.

EPA 1998. Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC): Final report. Environmental Protection Agency.

Fellenberg, K., Hauser, N., Brors, B., Neutzner, A., Hoheisel, J., Vingron, M., 2001. Correspondence analysis applied to microarray data. Proc. Natl. Acad. Sci. U.S.A. 98, 10781–10786.

Forrest, D., Reh, T., Ruesch, A., 2002. Neurodevelopmental control by thyroid hormone receptors. Curr. Opinion. Neurobiol. 12, 49–56.

Greenacre, M., 1984. Theory and applications of correspondence analysis. Academic Press, London.

Greenacre, M., 1993. Correspondence analysis in practice. Academic Press, London.

Helbing, C., Ji, L., Veldhoen, N., Zhang, F., Holcombe, G., Kosian, P., Tietge, J., Degitz, S., 2007. Identification of gene expression biomarkers that rapidly detect exposure to thyroid hormones, methimazole, propylthiouracil and perchlorate in a *Xenopus laevis* metamorphosis screening assay. Part 2. Effects on the tail and hindlimb. Aquat. Toxicol. 82, 215–226.

Helbing, C., Werry, K., Crump, D., Domanski, D., Veldhoen, N., Bailey, C., 2003. Expression profiles of novel thyroid hormone-responsive genes and proteins in the tail of *Xenopus laevis* tadpoles undergoing precocious metamorphosis. Mol. Endocrinol. 17, 1395–1409.

Helbing, C.C., Ovaska, K., Ji, L., 2006. Evaluation of the effect of acetochlor on thyroid hormone receptor gene expression in the brain and behavior of *Rana catesbeiana* tadpoles. Aquat. Toxicol. 80, 42–51.

Huang, H., Marsh-Armstrong, N., Brown, D., 1999. Metamorphosis is inhibited in transgenic *Xenopus laevis* tadpoles that over express type III deiodinase. Proc. Natl. Acad. Sci. U.S.A. 96, 962–967.

Jones, I., Forrest, D., 2003. The thyroid hormone receptor β gene: structure and functions in the brain and sensory systems. Thyroid 13, 1057–1068.

Kawahara, A., Gohda, Y., Hikosaka, A., 1999. Role of type III iodothyronine 5-deiodinase gene expression in temporal regulation of *Xenopus* metamorphosis. Dev. Growth Differ. 41, 365–373.

Krain, L., Denver, R., 2004. Developmental expression and hormonal regulation of glucocorticoid and thyroid hormone receptors during metamorphosis in *Xenopus laevis*. J. Endocrinol. 181, 91–104.

Montgomery, D. (Ed.), 1991. Introduction to Statistical Quality Control. Wiley, Hoboken, NJ, p. 204.

Nieuwkoop, P.D., Faber, J., 1956. Normal table of *Xenopus laevis*. North Holland Publishing, Amsterdam.

Opitz, R., Hartmann, S., Blank, T., Braunbeck, T., Lutz, I., Kloas, W., 2006. Evaluation of histological and molecular endpoints for enhanced detection of thyroid system disruption in *Xenopus laevis* tadpoles. Tox. Sci. 90, 337–348.

Sachs, L.M., Amano, T., Shi, Y.B., 2001. An essential role of histone deacetylases in postembryonic organ transformations in *Xenopus laevis*. Int. J. Mol. Med. 8, 595–601.

Sap, J., Munoz, A., Damm, K., Goldberg, Y., Bhysdael, J., Leutz, A., Beug, H., Vennstrom, B., 1986. The c-erbA protein is a high affinity receptor for thyroid hormone. Nature 324, 635–640.

Shi, Y.-B., 2000. Amphibian Metamorphosis: from morphology to molecular biology. Wiley-Liss, New York.

Shi, Y.-B., Hayes, W.P., 1994. Thyroid hormone-dependent regulation of the intenstinal fatty acid-binding protein gene during amphibian metamorphosis. Devel. Biol. 161, 48–58.

Siesser, W.B., Cheng, S.-Y., McDonald, M., 2005. Hyperactivity, impaired learning on a vigilance task, and a differential response to methylphenidate in the TRβPV knock-in mouse. Psychopharmacology 181, 653–663.

Soldin, O.P., Braverman, L.E., Lamm, S.H., 2001. Perchlorate clinical pharmacology and human health: a review. Ther. Drug Monitor. 23, 316–331.

Tan, Q., Brusgaard, K., Kruse, A., Oakeley, E., Hemmings, B., Beck-Nielsen, H., Hansen, L., Gaster, M., 2004. Correspondence analysis of microarray time-course data in case-control design. J. Biomed. Informatics 37, 358–365.

Tietge, J.E., Holcombe, G.W., Flynn, K.M., Kosian, P.A., Korte, J.J., Anderson, L.E., Wolf, D.C., Degitz, S.J., 2005. Metamorphic inhibition of *Xenopus laevis* by sodium perchlorate: effects on development and thyroid histology. Environ. Tox. Chem. 24, 926–933.

Turque, N., Palmier, K., Le Mevel, S., Alliot, C., Demeneix, B., 2005. A rapid, physiologic protocol for testing transcriptional effects of thyroid-disrupting agents in permetamorphic *Xenopus* tadpoles. Enivron. Health Persp. 113, 1588–1593.

Urbansky, E.T., 1998. Perchlorate chemistry: implications for analysis and remediation. Bioremed. J. 2, 81–95.

Veldhoen, N., Boggs, A., Walzak, K., Helbing, C.C., 2006a. Exposure to tetrabromobisphenol-A alters TH-associated gene expression and tadpole metamorphosis in the Pacific Tree Frog *Pseudacris regilla*. Aquat. Toxicol. 78, 292–302.

Veldhoen, N., Skirrow, R., Ji, L., Domanski, D., Bonfield, E., Bailey, C., Helbing, C., 2006b. Use of heterologous cDNA arrays and organ culture in the detection of thyroid hormone-dependent responses in a sentinel frog *Rana catesbeiana*. Comp. Biochem. Physiol. D 1, 187–199.

Veldhoen, N., Skirrow, R., Osachoff, H., Wigmore, H., Clapson, D., Gunderson, M., van Aggelen, G., Helbing, C., 2006c. The bactericidal agent Triclosan modulates thyroid hormone-associated gene expression and disrupts postembryonic anuran development. Aquat. Toxicol. 80, 217–227.

Wagner, M., Helbing, C., 2005. Multiple variants of the ING1 and ING2 tumor suppressors are differentially expressed and thyroid hormone-responsive in *Xenopus laevis*. Gen. Comp. Endocrinol. 144, 38–50.

Weinberger, C., Thompson, C., Ong, E., Lebo, R., Gruol, D., Evans, R., 1986. The c-erbA gene encodes a thyroid hormone receptor. Nature 324, 641–646.

Wolff, J., 1998. Perchlorate and the thyroid gland. Pharm. Rev. 50, 89–105.

Zhang, F., Degitz, S.J., Holcombe, G.W., Kosian, P.A., Tietge, J., Veldhoen, N., Helbing, C.C., 2006. Evaluation of gene expression endpoints in the context of a *Xenopus laevis* metamorphosis-based bioassay to detect thyroid hormone disruptors. Aquat. Toxicol. 76, 24–36.

Zoeller, R., 2001. Maternal thyroid hormone affects fetal brain development. Neurotoxicology 22, 131–132.

Zoeller, R., 2005. Environmental chemicals as thyroid hormone analogues: New studies indicate that thyroid hormone receptors are targets of industrial chemicals. Mol. Cell. Endocrinol. 242, 10–15.

Zoeller, R.T., Crofton, K.M., 2000. Thyroid hormone action in fetal brain development and potential for disruption by environmental chemicals. Neurotoxicology 23, 935–946.